PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 25 February 2000 (25.02.00)	
International application No. PCT/GB99/02317	**Applicant's or agent's file reference** IPD/P1209/WOD
International filing date (day/month/year) 19 July 1999 (19.07.99)	**Priority date** (day/month/year) 23 July 1998 (23.07.98)
Applicant DREWE, Lisa, Joanne et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

03 February 2000 (03.02.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Olivia RANAIVOJAONA
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

09 AUG 2000

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

BOWDERY A.O.
D/IPR
Formalities Section (DERA)
Poplar 2
MOD Abbey Wood No 19
Bristol BS34 8JH
GRANDE BRETAGNE

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing *(day/month/year)*	04.08.2000
Applicant's or agent's file reference P1209/WOD	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/02317	19/07/1999	23/07/1998

Applicant
THE SECRETARY OF STATE FOR DEFENCE ... et al

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Digiusto, M Tel.+49 89 2399-8162

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREA

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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
IPD/P1209/WOD		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 99/ 02317	19/07/1999	23/07/1998
Applicant		
THE SECRETARY OF STATE FOR DEFENCE ...et,al		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[X] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

[ ] the text is approved as submitted by the applicant.

[X] the text has been established by this Authority to read as follows:

NUCLEIC ACID DETECTION METHOD BY TRIPLE HELIX FORMATION

5. With regard to the **abstract**,

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/02317

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 92 11390 A (IDEXX LAB INC) 9 July 1992 (1992-07-09) page 10, line 7 -page 11 page 34, paragraph 1; figure 1	1-19
Y	WO 97 14793 A (UNIV BOSTON) 24 April 1997 (1997-04-24) page 5, line 11 - line 17 page 13 -page 14, paragraph 1	1-19

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 November 1999	18/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Osborne, H

3

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SEEGER C ET AL: "PNA-MEDIATED PURIFICATION OF PCR AMPLIFIABLE HUMAN GENOMIC DNA FROMWHOLE BLOOD" BIOTECHNIQUES, vol. 23, no. 3, 1 September 1997 (1997-09-01), pages 512-514, 516/517, XP000703351 ISSN: 0736-6205 the whole document	1-19
Y	J WANG ET AL: "Peptide nucleic acids probes for sequence-specific DNA biosensors" JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 118, no. 33, 21 August 1996 (1996-08-21), pages 7667-7670, XP002094817 ISSN: 0002-7863 the whole document	10,11, 16,17
Y	KAI E ET AL: "Novel DNA detection system of flow injection analysis (2): The distinctive properties of a novel system employing PNA (peptide nucleic acid) as a probe for specific DNA detection" NUCLEIC ACIDS SYMPOSIUM SERIES, vol. 37, 1997, pages 321-2, XP002121513 the whole document	10,11, 16,17

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/02317

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9211390 A	09-07-1992	EP 0566670 A	27-10-1993
		US 5800984 A	01-09-1998
WO 9714793 A	24-04-1997	AU 7016096 A	07-05-1997

PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P1209/WOD		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/02317	19/07/1999	23/07/1998
International Patent Classification (IPC) or national classification and IPC C12Q1/68		
Applicant THE SECRETARY OF STATE FOR DEFENCE ... et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
03/02/2000	04.08.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Maucher, C Telephone No. +49 89 2399 7415

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/02317

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-8 as originally filed

Claims, No.:

1-17 with telefax of 06/07/2000

Drawings, sheets:

1/2,2/2 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 17.

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 17 are so unclear that no meaningful opinion could be formed (*specify*):

see separate sheet

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-16
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-16
Industrial applicability (IA)	Yes:	Claims	1-16
	No:	Claims	

2. Citations and explanations

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Point III:

Claim 17 could not be examined, since the scope of the claim is not defined due to the lack of any technical feature (see also point VIII).

Point V:

The applicant's observations submitted with the amended claims (with a letter dated 4.7.2000) have been considered in establishing this report.

The following documents have been considered for the purposes of this report:

D1: WO-A-92 11390
D2: WO-A-97 14793
D3: BIOTECHNIQUES,
vol. 23, no. 3, 1997, pages 512-514, 516/517
D4: NUCLEIC ACIDS SYMPOSIUM SERIES,
vol. 37, 1997, pages 321-322
D5: BIOSENSORS AND BIOELECTRONICS,
vol. 14, 1999, pages 397-404

D5 has been submitted by the applicant as an enclosure to his letter of reply dated 4.7.2000 to the written opinion dated 5.4.2000.

1. Article 33(2) PCT

1.1. The subject-matter of **claims 1-13** is novel, since none of the available prior art documents discloses a method for detecting the presence of a target nucleic acid in a sample, which is amplified by PCR, and wherein triplex structures of PNA and DNA are detected subsequently.

1.2. The subject-matter of **claims 14-16** is novel, since none of the available prior art documents discloses a kit comprising PNA and a primer with a plurality of pyrimidine residues at the 5' region thereof.

2. Article 33(3) PCT

2.1. The subject-matter of **claim 1** does not meet the requirements of Article 33(3) PCT for the following reasons.

D1, which is the closest available prior art, describes a method for detecting a nucleic acid, comprising amplifying said nucleic acid by PCR to yield product duplexes and detecting one of said duplexes by hybridizing a third strand of nucleic acid to said product duplexes without denaturation (claims 1 and 2). Triple helix forming regions, which are stretches of polypurine and polypyrimidine residues (page 7, lines 4-9) may be incorporated into the target sequence during amplification, e.g. by PCR (page 10, lines 8-14). D1 is distinguished from the subject-matter of claim 1 in that no PNA is used.

Thus, the technical problem to be solved in the light of D1 is the provision of a nucleic acid detection method comprising triple helix formation wherein a specific nucleic acid is used.

The problem is solved by contacting the sample, which comprises a target nucleic acid, with PNA, that is able to bind to at least a portion of said target nucleic acid.

The skilled person, being aware of D1 and D2, would not need any inventive activity for providing a method as disclosed in D1 by using the nucleic acid PNA for triplex formation. D2 discloses nucleic acid clamps which are linear polymers comprising two PNA sequences which together can form a triple helix with a target nucleic acid (page 5, lines 12-14). In the PNA_2DNA triplex, one polypyrimidine PNA strand forms a base pairing with the polypurine nucleic acid (page 14, lines 4-7).

Moreover, the skilled person would also be prompted to use PNA in a nucleic acid detection method when being aware of D1 and D3. D3 reveals triplex formation of bisPNA with a DNA target (page 513, last column, 1. full paragraph) for purification of the target (abstract).

The features of dependent **claims 2-9 and 12**, as well as independent **claim 13**, have already been employed for the same purpose in similar methods (D1, D2 and/or D3) to the one disclosed in the present application. It would therefore be obvious (Article 33(3) PCT) to the person skilled in the art, to apply these features with corresponding effect to an alternative method according to document D1, thus arriving at a method according to claims 2-9 and 12 and the use of claim 13:

- **claim 2** (D3, page 513, last column, 1. full paragraph)
- **claims 3-4** (D1, figure 1)
- **claim 5** (D1, claim 2; D2, page 17, line 15)
- **claim 6** (D1, page 7, lines 4-9, page 12, lines 13-17; D2, page 14, lines 4-7)
- **claim 7** (D1, page 7, lines 4-9 and page 10, lines 8-14)
- **claim 8** (D1, sequences disclosed on page 16: pyrimidines are C and T)
- **claim 9** (D1, claim 12)
- **claim 12** (D1, page 18, lines 4-5 from the bottom; D2, page 22, lines 11-12; D3, figure 2: electrophoresis)
- **claim 13** (D1: primers comprising a plurality of pyrimidine residues at the 5' end (sequences on page 16 and lines 12-14) in the triple helical detection of PCR-amplified nucleic acid target sequences (page 15, lines 5-6 from the bottom)

2.2. **Claims 10-11** contain only features common in the art (D4, abstract) and are therefore not inventive according to Article 33(3) PCT.

2.3. The combination of the teaching of D1 (see below) and D2 or D3 (both disclose PNA for triplex formation with DNA; see 2.1.) renders the subject-matter of **claims 14-16** obvious (Article 33(3) PCT). In D1, a kit is disclosed comprising a single-stranded nucleic acid probe capable of specifically hybridizing to a triple-helix forming sequence of a target nucleic acid (claim 50). Said kit can additionally comprise a primer (claim 59). Primers comprising a plurality of pyrimidine residues at the 5' end are also disclosed in D1 (see the sequences on page 16, and lines 12-14). A skilled person does not need any inventive activity for providing a kit as disclosed in D1 with PNA (D2, D3; see abstracts and claim 30 of D2) as nucleic acid probe when being aware of the methods and kits disclosed in D1 and D2 or D3.

Point VII:

1. The key of figure 2 is not clear, since the identical symbol (an empty bar) is used for the target sequence, the sequence complementary to the target, as well as for the 5'-polypyrimidine tail (CT).

2. The abbreviations "L" and "N" (page 7, lines 1 and 12) have not been explained at least once in the patent specification.

3. Contrary to the requirements of Rule 5.1(a)(ii) PCT, the documents D1-D4 have not been identified in the description and the relevant background art disclosed therein has not been briefly discussed.

Point VIII:

1. Claim 1 (method step (b)) does not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined. Method step (b) of said claim attempts to define the subject-matter in terms of the result to be achieved which merely amounts to a statement of the underlying problem. The technical features necessary for achieving this result should be added.

2. The subject-matter of claim 13 is not clear (Article 6 PCT), since it cannot unambiguously be derived from the wording of the claim that the pyrimidine residues shall be part of the primer sequence (see page 5 of the present description, lines 18-20) and not of the target nucleic acid sequence.

3. Claim 17 is unclear (Article 6 PCT), since it does not contain any technical feature. Furthermore, claim 17 contains references to the description and the drawings. According to Rule 6.2(a) PCT, claims should not contain such references except where absolutely necessary, which is not the case here.

Claims

1. A method for detecting the presence of a target nucleic acid sequence in a sample, said method comprising

(a) amplifying said target nucleic acid so that the product of the amplification reaction includes a purine rich region,

(b) contacting the sample with a peptide nucleic acid able to bind to at least a portion of said target sequence; and

(c) detecting the presence of triplex DNA structures.

2. A method according to claim 1 wherein the peptide nucleic acid is bis-PNA.

3. A method according to claim 1 or claim 2 wherein the amplification product is exposed to the peptide nucleic acid during or after the amplification reaction.

4. A method according to claim 3 wherein the amplification product is exposed to the peptide nucleic acid after completion of the amplification reaction.

5. A method according to any one of the preceding claims wherein the amplification reaction is a polymerase chain reaction.

6. A method according to any one of the preceding claims wherein the target nucleic acid contains a purine rich region.

7. A method according to any one of the preceding claims wherein a purine rich region is introduced into the amplification product during the amplification reaction.

8. A method according to claim 7 wherein primers used in the amplification comprise a plurality of pyrimidines at the 5' end thereof.

9. A method according to any one of the preceding claims wherein the peptide nucleic acid is immobilised on a support.

10. A method according to claim 9 wherein the support is a waveguide of a detection device.

11. A method according to claim 10 wherein the detection device is a surface plasmon resonance detector.

12. A method according to any one of claims 1 to 8 wherein the triplex structure is detected by a gel retardation method.

13. A primer comprising a sequence which hybridises to an end region of a target nucleic acid sequence, and a plurality of pyrimidine residues at a 5'region thereof.

15. A kit for carrying out a method according to any one of the preceding claims, said kit comprising a peptide nucleic acid sequence which is specific for a target nucleotide sequence.

16. A kit according to claim 14 wherein the peptide nucleic acid is immobilised on a waveguide of an evanescent wave detector apparatus.

17. A kit according to claim 15 wherein the evanescent wave detector apparatus is a surface plasmon resonance detector.

18. A kit according to any one of claims 15 to 17 which further comprises a primer according to claim 13.

19. A method for detecting a nucleotide sequence according to claim 1, substantially as hereinbefore described.

Claims

1. A method for detecting the presence of a target nucleic acid sequence in a sample, said method comprising

(a) amplifying said target nucleic acid so that the product of the amplification reaction includes a purine rich region;

(b) contacting the sample with a peptide nucleic acid able to bind at least a portion of said target sequence; and

(c) detecting the presence of triplex structures.

2. A method according to claim 1 wherein the peptide nucleic acid is bis-PNA.

3. A method according to claim 1 or claim 2 wherein the amplification product is exposed to the peptide nucleic acid during or after the amplification reaction.

4. A method according to claim 3 wherein the amplification product is exposed to the peptide nucleic acid after completion of the amplification reaction.

5. A method according to any one of the preceding claims wherein the amplification reaction is a polymerase chain reaction.

6. A method according to any one of the preceding claims wherein the target nucleic acid contains a purine rich region.

7. A method according to any one of claims 1 to 5 wherein a purine rich region is introduced into the amplification product during the amplification reaction.

8. A method according to claim 7 wherein primers used in the amplification comprise a plurality of pyrimidines at the 5' end thereof.

9. A method according to any one of the preceding claims wherein the peptide nucleic acid is immobilised on a support.

10. A method according to claim 9 wherein the support is a waveguide of a detection device.

11. A method according to claim 10 wherein the detection device is a surface plasmon resonance detector.

12. A method according to any one of claims 1 to 8 wherein the triplex structure is detected by a gel retardation method.

13. The use of a primer comprising a sequence which hybridises to an end region of a target nucleic acid sequence, and a plurality of pyrimidine residues at a 5'region thereof; in a method according to any one of the preceding claims.

14. A kit for carrying out a method according to any one of the preceding claims, said kit comprising a peptide nucleic acid sequence which is specific for a target nucleotide sequence, and a primer comprising a sequence which hybridises to an end region of a target nucleic acid sequence, and a plurality of pyrimidine residues at a 5'region thereof.

15. A kit according to claim 14 wherein the peptide nucleic acid is immobilised on a waveguide of an evanescent wave detector apparatus.

16. A kit according to claim 15 wherein the evanescent wave detector apparatus is a surface plasmon resonance detector.

17. A method for detecting a nucleotide sequence according to claim 1, substantially as hereinbefore described.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12Q 1/68 — A1

(11) International Publication Number: **WO 00/05408**

(43) International Publication Date: 3 February 2000 (03.02.00)

(21) International Application Number: PCT/GB99/02317

(22) International Filing Date: 19 July 1999 (19.07.99)

(30) Priority Data:
9815933.8 23 July 1998 (23.07.98) GB

(71) Applicant *(for all designated States except US)*: THE SECRETARY OF STATE FOR DEFENCE [GB/GB]; Defence Evaluation and Research Agency, Ively Road, Farnborough, Hampshire GU14 0LX (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: DREWE, Lisa, Joanne [GB/GB]; CBD Porton Down, Salisbury, Wiltshire SP4 0JQ (GB). BRIGHTWELL, Gale [GB/GB]; CBD Porton Down, Salisbury, Wiltshire SP4 0JQ (GB). HALL, Elizabeth, Ann, Howlett [GB/GB]; CBD Porton Down, Salisbury, Wiltshire SP4 0JQ (GB).

(74) Agent: BOWDERY, A., O.; D/IPR, Formalities Section, Poplar 2, MOD Abbey Wood #19, Bristol BS34 8JH (GB).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: NUCLEIC ACID DETECTION METHOD BY TRIPLE HELIX FORMATION

(57) Abstract

A method for detecting the presence of a target nucleic acid sequence in a sample, said method comprising (a) amplifying said target nucleic acid so that the product of the amplification reaction includes a purine rich region, (b) contacting the sample with a peptide nucleic acid able to bind to at least a portion of said target sequence; and (c) detecting the presence of triplex DNA structures. The detection is suitably effected directly, for example using a surface plasmon resonance detector.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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NUCLEIC ACID DETECTION METHOD BY TRIPLE HELIX FORMATION

The present invention relates to a method of detecting specific target DNA sequences, and in particular to the products of amplification reactions, as well as to reagents and apparatus used in that method.

Many methods are known in order to detect the presence of particular target DNA sequences in a sample. A substantial proportion of these methods require that the DNA is denatured to single stranded form and then this sequence is hybridised or otherwise allowed to bind to a labelled sequence specific probe.

The target sequences are frequently subjected to amplification reactions, for example the polymerase chain reaction or the ligase chain reaction, in order to increase the amount of the target sequence to detectable levels.

Other methods of detecting sequences include the use of intercalating dyes which are incorporated into the sequences during the amplification reaction. However such methods are relatively non specific as the dyes will intercalate with any amplification product, even if they are the result of non-specific amplification products.

Other assays such as the TAQMAN™ assay utilise complex probes which include reporter and quencher moieties during the course of the amplification process. These probes hybridise to single stranded target sequences during the amplification reaction and are then digested by the enzymes carrying out the reaction. The relationship between quencher and reporter molecule of the probe produces a signal which can be monitored. The probes used in this case however, are complex and expensive.

It is known that peptide nucleic acids will strand invade DNA at purine rich sites to form triplex structures (P.E. Nielson et al., Science, 1991, 254, p1497-1506, Turney D.Y. et al,. Proc. Natl. Acad. Sci. USA , 1993, 90, 1667-1670). The mechanism by which this is effected is illustrated diagrammatically hereinafter in Figure 1.

The applicants have found that this phenomenon can be used in detection of target DNA sequences.

A method for detecting the presence of a target nucleic acid sequence in a sample, said method comprising

(a) amplifying said target nucleic acid so that the product of the amplification reaction includes a purine rich region,

(b) contacting the sample with a peptide nucleic acid able to bind to at least a portion of said target sequence; and

(c) detecting the presence of triplex DNA structures.

The method enabling the direct detection of target sequences, for example amplification products without the usual denaturation step required for duplex formation with a nucleic acid probe.

The expression "purine rich region" means that the sequence is suitable for strand invasion by a peptide nucleic acid (PNA). Such regions suitably contain at least four consecutive purine residues.

The reaction in step (a) above is suitably effected in the presence of a buffer, and preferably a low salt buffer for example containing 50mM or less of salt as this favours triplex formation as compared to DNA:DNA duplexes. Furthermore, the pH of the buffer used will depend on the precise nature of the PNA

employed. If C's are used in the PNA strand to strand invade G's on duplex DNA, careful consideration has to be given to the pH of the buffer as the C involved in forming the Hoogsteen base-pair needs to be protonated, requiring a buffer of low pH, for example of less than 4.5.

The peptide nucleic acid used in the method of the invention may be single stranded or it may be bis-PNA. Preferably, the peptide nucleic acid used in the method is a bis-PNA as this results is a faster strand invasion process and a more stable triplex product.

Bis-PNA will comprise of two anti-parallel strands joined by a hydrophilic linker. One strand will be designed for Watson-Crick recognition of DNA within the target sequence, and the other strand is designed for Hoogsteen recognition of a PNA-DNA duplex. Such acids will be optimal for PNA_2DNA triplex stability and thus enhance strand-displacement binding to double-stranded DNA.

Peptide nucleic acids used will suitably contain a sequence of poly-T's or poly-C's.

The target nucleic acid is first subject to an amplification reaction such as the polymerase chain reaction (PCR) or ligase chain reaction (LCR), preferably PCR. The product may be exposed to the peptide nucleic acid during or after the amplification reaction, but is preferably exposed to the peptide nucleic acid after completion of the amplification reaction.

Where the target nucleotide sequence contains or is selected such that it contains a purine rich region, the method can be carried out directly. Where such regions do not exist in the target sequence, they may be introduced during the amplification

reaction. In this case, the amplification will be effected using one or more primers which comprise a plurality of pyrimidines, suitably at the 5′ end thereof. This region will chain extend during the extension phase of the amplification (as illustrated in Figure 2 hereinafter). The 3′-end of both amplified strands of the amplification obtained using these primers should now contain the purine rich sites. Indeed, PCR products, that were tagged in this manner, have been cloned and sequenced and were found to have the poly-purine stretches incorporated at their 3′end. This ensures that a suitable PNA binding purine rich region is contained within the amplification product.

Primers of this sort form a further aspect of the invention.

The triplex formed may be detected using various methods in step (b). For example, gel retardation methods may be used. When the product is subjected to gel electrophoresis, for instance on a non-denaturing polyacrylamide gel, and then stained using conventional reagents such as ethidium bromide, the presence of a retarded triplex fraction can be observed.

This method however is relatively slow. Furthermore, comparison with a similar sequence which is not in the form of a triplex is required as a standard.

Preferably therefore, the detection is effected using a capture assay. The capture agent in this case is suitably the PNA sequence which is immobilised on a support. The sample is then contacted with the support whereupon any target sequence present will become associated with the PNA on the surface. It can then be detected using any of the known techniques.

In a particularly preferred embodiment, the support is a waveguide of a detection device which operates using evanescent wave detection. An example of such a device is a surface plasmon resonance detector. This allows the direct and rapid detection of target nucleotide sequence within a sample.

Thus a product of the amplification reaction is simply allowed to flow over the waveguide of such a detector and the presence of an amplicon can be detected in something approaching "real time".

In a further aspect, the invention provides a kit for use in the method of the invention. These kits suitably comprise a PNA designed to form a triplex with a target DNA. Optionally also, it may contain primers which can be used in the amplification of the target DNA, in particular primers which are 5'-tagged with pyrimidines.

The kit may also comprise a waveguide of a evanescent wave detector and particularly a surface plasmon resonance detector having supported thereon, the peptide nucleic acid which specifically binds a target DNA sequence.

The invention will now be particularly described by way of example with reference to the accompanying diagrammatic drawings in which:

Figure 1 illustrates diagrammatically PNA:DNA triplex formation;

Figure 2 illustrates diagrammatically the incorporation of purine rich regions into an amplification product, using 5'-tagging of primers with polyramidine sequences; and

Figure 3 illustrates triplex formation on the surface of a surface plasmon resonance detector.

Example 1

Triplex Formation

The ability of PNA to form triplex structures with PCR products has been demonstrated using gel retardation studies. Two PCR products were chosen for study. One has a sequence capable of forming triplexes with a PNA probe i.e. contains poly-A sites.

PCR82

5'

ATAAATACAACCAACAAAATAAATAGTCATAAAATTGTATACATTAGCAATGCATACC ACAAAGTTCTAAGTACTAAAATAT 3' (SEQ ID NO 1)

The other does not contain poly-A sites and acts as a negative control.

PCR 175

5'

GCGAAACGGAACATAGCCCAAACCAAGAGGCTTGCCTCTTGGGGTTGTAGGACATTCT ATACGGAGTTACAAAGGAAGCAGGTAGACGAAGCGACCTGGAAAGGTCCGTCGTAGAGGGTAAC AACCCCGTAGTCGAAACTTCGTTCTCTCTTGAATGTATCCTGAGTACGGCGGAACACGTGAAA 3' (SEQ ID NO 2)

Two types of PNA probe were used, one was a linear sequence and contains a sequence of poly-T's

PNA057

N TTTTCCTTCCCTTTT C (SEQ ID NO 3)

The other, a bis-PNA of the same linear sequence but composed of two anti-parallel strands joined by a hydrophilic linker. One

strand was designed for Watson-Crick recognition of DNA and the other strand is designed for Hoogsteen recognition of a PNA-DNA duplex and should be optimal for PNA_2DNA triplex stability and thus enhance strand-displacement binding to double-stranded DNA.

PNA058

N TTTTCCCTTCCTTTT LLL TTTTCCTTCCCTTTT C (SEQ ID NO 4)

Each PCR product (5 µg/ml) was incubated with each PNA probe (10 µg/ml), at 37°C in 0.5 X TE buffer (1 mM Tris.HCl, 0.1 mM EDTA, 5 mM NaCl, pH 8.0) for varying time intervals before the reaction was terminated by adding 150 mM HBS, pH 7.4 on ice. Samples were run on a non-denaturing 12% polyacrylamide gel. The electrophoretic mobility of the triplex PNA_2DNA was compared to the duplex DNA of the relevant PCR product and visualised by EtBr staining. Triplex structures were observed suggesting that PNA can directly detect double-stranded PCR products.

The results of the gel retardation studies showed that single-stranded PNA did not strand invade the PCR products within the first 60 minutes. (This is backed up in the literature where it has been demonstrated that the association of a bis-PNA with a single strand of homopurine DNA gives a complex that is significantly more stable than the one formed with two single PNA strands due to a more favourable entropy of reaction.)

Bis-PNA, however, formed a triplex within the first 10 minutes of reaction.

Example 2

Detection of triplexes on a surface plasmon resonance (SPR) surface.

Biotin labelled bis-PNA (50 µg/ml) was linked to a dextran surface (Biacore, SAchip) via a streptavidin-biotin interaction. A sample of both PCR products (10 µg/ml), in water, was flowed

over this sensor surface and were detected by a change in refractive index. The SPR system could differentiate between purine-rich and non-purine rich PCR products in near real time (See Figure 3).

Claims

1. A method for detecting the presence of a target nucleic acid sequence in a sample, said method comprising

(a) amplifying said target nucleic acid so that the product of the amplification reaction includes a purine rich region,

(b) contacting the sample with a peptide nucleic acid able to bind to at least a portion of said target sequence; and

(c) detecting the presence of triplex DNA structures.

2. A method according to claim 1 wherein the peptide nucleic acid is bis-PNA.

3. A method according to claim 1 or claim 2 wherein the amplification product is exposed to the peptide nucleic acid during or after the amplification reaction.

4. A method according to claim 3 wherein the amplification product is exposed to the peptide nucleic acid after completion of the amplification reaction.

5. A method according to any one of the preceding claims wherein the amplification reaction is a polymerase chain reaction.

6. A method according to any one of the preceding claims wherein the target nucleic acid contains a purine rich region.

7. A method according to any one of the preceding claims wherein a purine rich region is introduced into the amplification product during the amplification reaction.

8. A method according to claim 7 wherein primers used in the amplification comprise a plurality of pyrimidines at the 5' end thereof.

9. A method according to any one of the preceding claims wherein the peptide nucleic acid is immobilised on a support.

10. A method according to claim 9 wherein the support is a waveguide of a detection device.

11. A method according to claim 10 wherein the detection device is a surface plasmon resonance detector.

12. A method according to any one of claims 1 to 8 wherein the triplex structure is detected by a gel retardation method.

13. A primer comprising a sequence which hybridises to an end region of a target nucleic acid sequence, and a plurality of pyrimidine residues at a 5'region thereof.

15. A kit for carrying out a method according to any one of the preceding claims, said kit comprising a peptide nucleic acid sequence which is specific for a target nucleotide sequence.

16. A kit according to claim 14 wherein the peptide nucleic acid is immobilised on a waveguide of an evanescent wave detector apparatus.

17. A kit according to claim 15 wherein the evanescent wave detector apparatus is a surface plasmon resonance detector.

18. A kit according to any one of claims 15 to 17 which further comprises a primer according to claim 13.

19. A method for detecting a nucleotide sequence according to claim 1, substantially as hereinbefore described.

Fig.1.

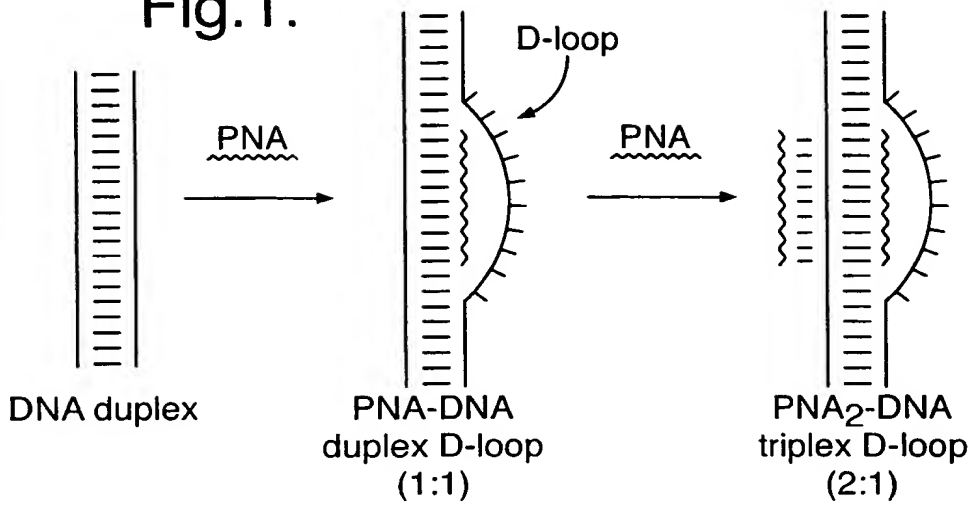

D-loop
PNA
PNA
DNA duplex
PNA-DNA
duplex D-loop
(1:1)
PNA2-DNA
triplex D-loop
(2:1)

Fig.3.

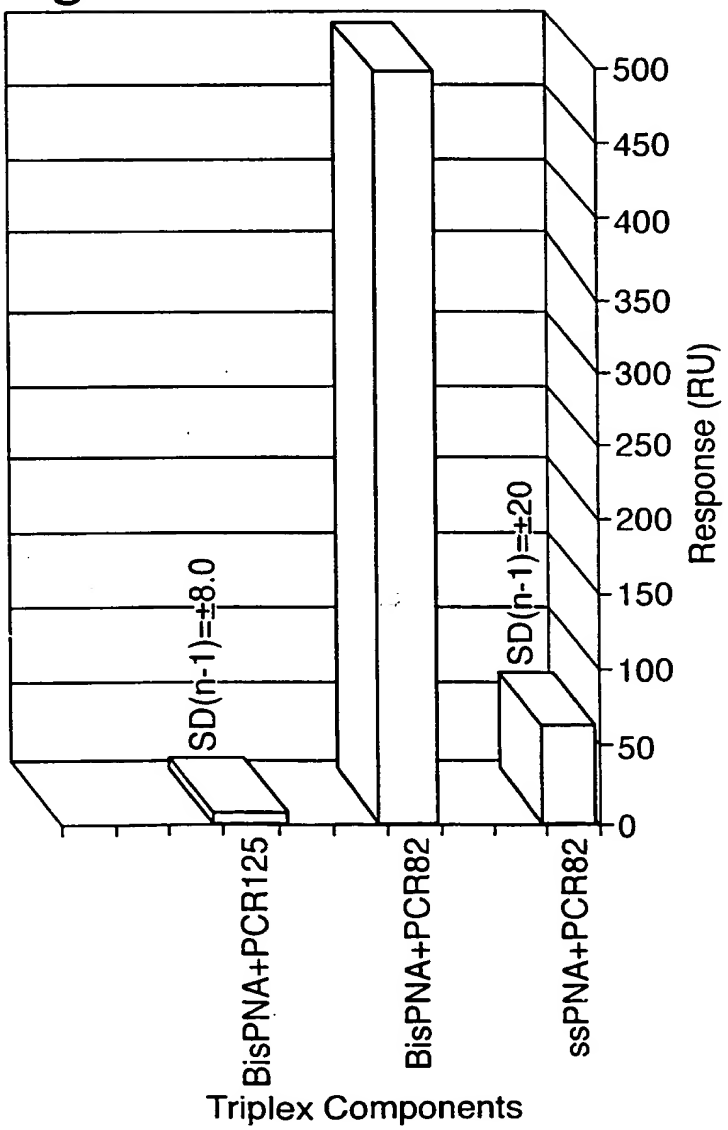
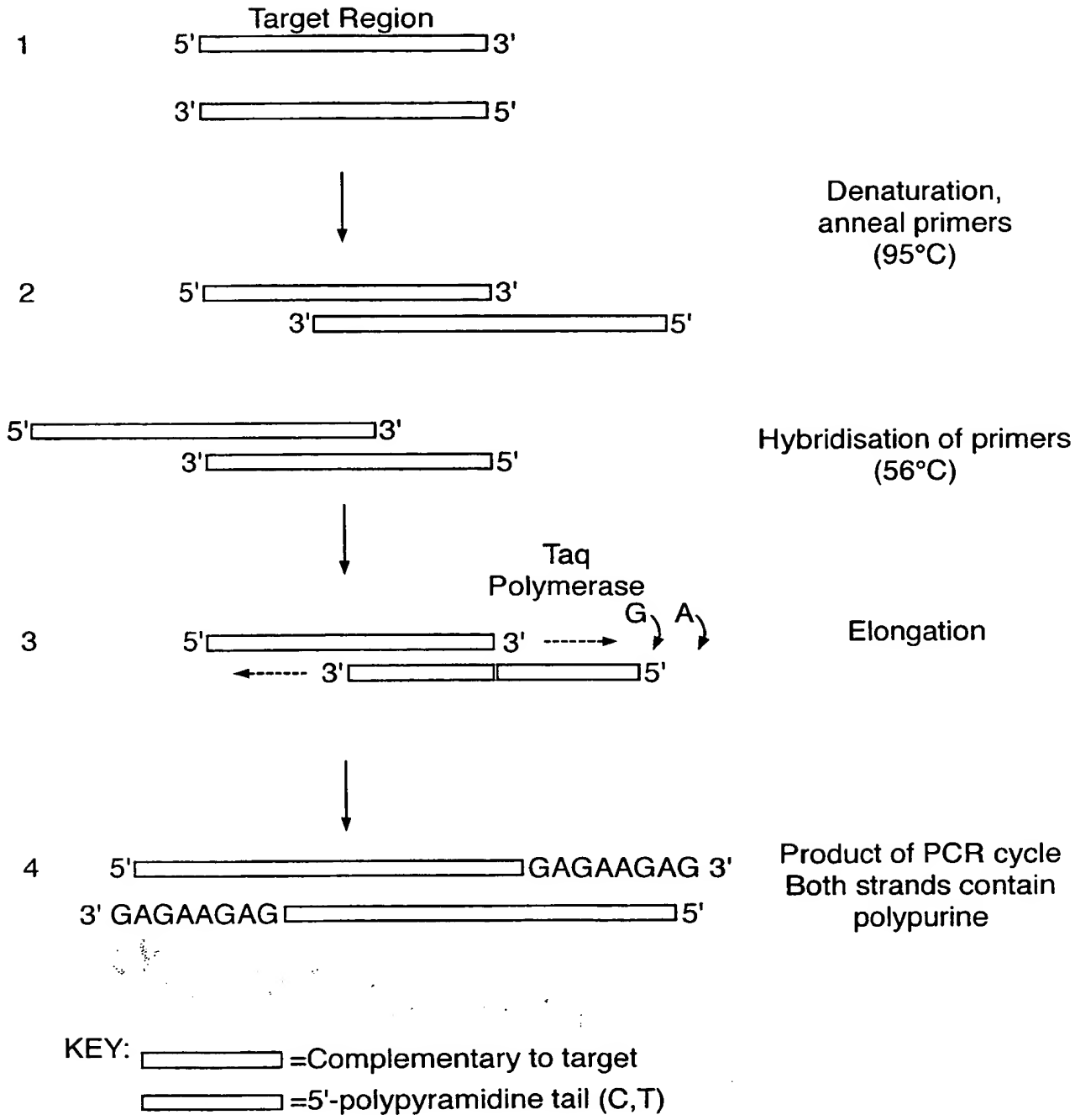

500
450
400
350
300
250
200
150
100
50
0
Response (RU)
SD(n-1)=±8.0
SD(n-1)=±20
BisPNA+PCR125
BisPNA+PCR82
ssPNA+PCR82
Triplex Components

Fig.2.


Target Region
1
5'
3'
3'
5'
Denaturation, anneal primers (95°C)
2
5'
3'
3'
5'
5'
3'
3'
5'
Hybridisation of primers (56°C)
Taq Polymerase
G
A
3
5'
3'
3'
5'
Elongation
4
5'
GAGAAGAG 3'
3' GAGAAGAG
5'
Product of PCR cycle Both strands contain polypurine
KEY:
=Complementary to target
=5'-polypyramidine tail (C,T)

INTERNATIONAL SEARCH REPORT

International Application No
PC./GB 99/02317

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 92 11390 A (IDEXX LAB INC) 9 July 1992 (1992-07-09) page 10, line 7 -page 11 page 34, paragraph 1; figure 1 ---	1-19
Y	WO 97 14793 A (UNIV BOSTON) 24 April 1997 (1997-04-24) page 5, line 11 - line 17 page 13 -page 14, paragraph 1 --- -/--	1-19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
4 November 1999	18/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Osborne, H

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate. of the relevant passages	Relevant to claim No.
Y	SEEGER C ET AL: "PNA-MEDIATED PURIFICATION OF PCR AMPLIFIABLE HUMAN GENOMIC DNA FROMWHOLE BLOOD" BIOTECHNIQUES, vol. 23, no. 3, 1 September 1997 (1997-09-01), pages 512-514, 516/517, XP000703351 ISSN: 0736-6205 the whole document	1-19
Y	J WANG ET AL: "Peptide nucleic acids probes for sequence-specific DNA biosensors" JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 118, no. 33, 21 August 1996 (1996-08-21), pages 7667-7670, XP002094817 ISSN: 0002-7863 the whole document	10,11, 16,17
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INTERNATIONAL SEARCH REPORT

[redacted]nation on patent family members

[redacted]national Application No
PCT/GB 99/02317

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		US 5800984 A	01-09-1998
WO 9714793 A	24-04-1997	AU 7016096 A	07-05-1997